

Automated 3D Cell Culture and Screening by Imaging and Flow Cytometry

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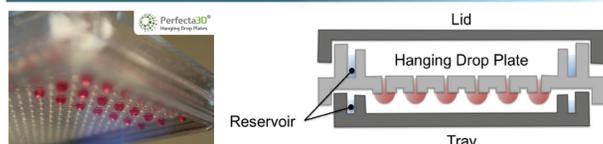
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Abstract

Drug developers are rapidly adopting three-dimensional (3D) cell cultures for drug screening since they provide a more physiologically relevant environment than two-dimensional cell cultures to screen compounds. Spheroids are a simple and well characterized, *in vitro* tumor model system and when derived from multiple cell types are increasingly being recognized as useful organoid models. Perfecta3D[®] Hanging Drop Plates (HDPs) facilitate the culture of consistent and controllable spheroids and co-cultures.

Manual manipulations of these spheroid cultures can be laborious and challenges are amplified as sample throughput increases. Automation can assist 3D cell culture laboratories by reducing the time spent on these manipulations while also improving their consistency. The Biomek FX^P Workstation was used to automate the culture and drug sensitivity screening of cancer spheroids in HDPs. Automated steps include the plating of cells and addition of compounds and staining reagents within the hanging drops for high content imaging. The transfer and dissociation of the spheroids into single cell suspensions was also automated to enable flow cytometry analysis. The Biomek FX^P Workstation utilized Enhanced Multichannel Selective Tip Pipetting for rapid multichannel liquid transfer to partial plates during the optimization phase of the workflow. By integrating additional components such as incubators and cell analyzers to the Biomek FX^P Workstation, the entire workflow can be automated in a scheduled fashion that further minimizes user interventions and helps manage overlapping experiments.

Materials and Methods



Cell Culture: Perfecta3D Hanging Drop plates were used to culture spheroids from HCT116 colon cancer cells. 4000 cells were plated in 40 μ l media containing 0.25% polyvinyl alcohol to accelerate the initial cellular aggregation. Various compounds were diluted and added to day 3 spheroids to induce apoptosis. After 24 hours, spheroids were assayed for apoptosis by staining with propidium iodide and CellEvent[®] Caspase-3/7 Green (Life Technologies). For imaging assays, cell nuclei were stained with NucBlue[®] Hoechst 33342 (Life Technologies).

High-Content Imaging: An ImageXpress[®] Micro (Molecular Devices) was used to acquire images of spheroids. For size and circularity images, day 3 spheroids were imaged under 10X magnification using transmitted light. Fluorescent images were acquired of day 4 spheroids after compound treatment using DAPI, FITC and Cy3 filters at 10X magnification. Pauses were added between filter changes to ensure the spheroid was stationary during image acquisition. Z-stacks were then acquired to ensure the optimal plane of focus for each image.

Flow Cytometry: Flow cytometry preparation required the transfer and subsequent dissociation of spheroids by addition of Accumax[®] and mechanical disruption through repeated pipetting. Dissociated cells were then stained and analyzed on a Gallios flow cytometer (Beckman Coulter). 2500 events were acquired per sample in FL1 and FL3 channels and data was analyzed in Kaluza software (Beckman Coulter).

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Automated 3D Culture and Analysis



Figure 2. Cell culture system. A) A Biomek FX^P with a 96-channel head and Span-8 pipettors was utilized for all 3D cell culture plating, compound dilution and addition, and sample preparation steps. A HEPA-filtered enclosure provided a sterile environment for cell manipulations and integrated storage and incubation devices enabled complete workflow automation. B) The 96-channel head utilized enhanced selective tip pipetting, which allows any pattern of tips to be used. This enables access of lower volume reservoirs and row-wise serial dilutions.

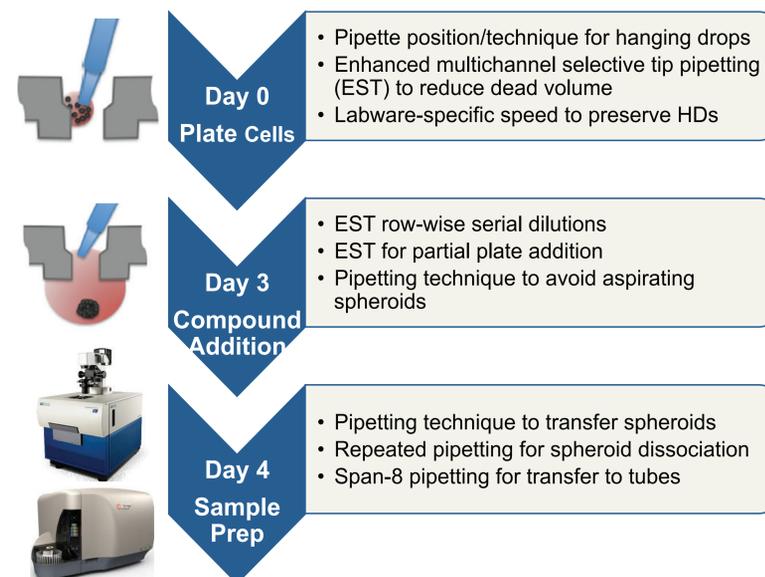
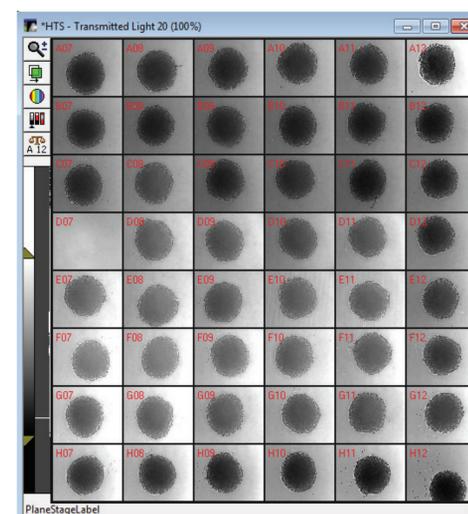


Figure 2. 3D Culture Workflow. The formation, treatment, and analysis of cancer spheroids required three steps over a four days process. Each of these steps presented unique challenges that were overcome through automation.

Spheroid Consistency



Measurement	Average	%CV
Area	187060	5.95
Perimeter	1661	2.86
Shape Factor	0.85	1.96

Figure 3. Spheroid Consistency. Day 3 spheroids were imaged and analyzed for size (area and perimeter) and circularity (shape factor). Across 47 images, the consistency of spheroids is illustrated by coefficients of variation (CVs) below 6%. Shape factor of 0.85 indicates excellent circularity as a perfect circle has a value of 1.0.

Apoptosis Analysis

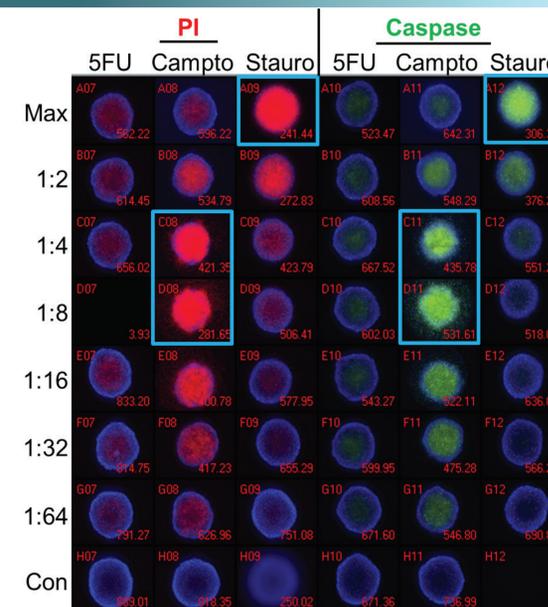


Figure 4. Apoptosis Analysis - Spheroids. Spheroids were treated with 5-fluorouracil (5FU), camptothecin (Campto), and staurosporine (Stauro) at the indicated dilutions for 24 hours and stained for apoptosis markers for analysis by imaging. Control spheroids (Con) were treated with DMSO alone. Wells with maximal staining by propidium iodide (PI) or caspase 3/7 substrate are identified by blue boxes. Staurosporine shows a traditional dose response while the highest level of staining was seen at 1:4-1:8 dilutions of camptothecin. 5-fluorouracil treatment resulted in minimal staining of spheroids.

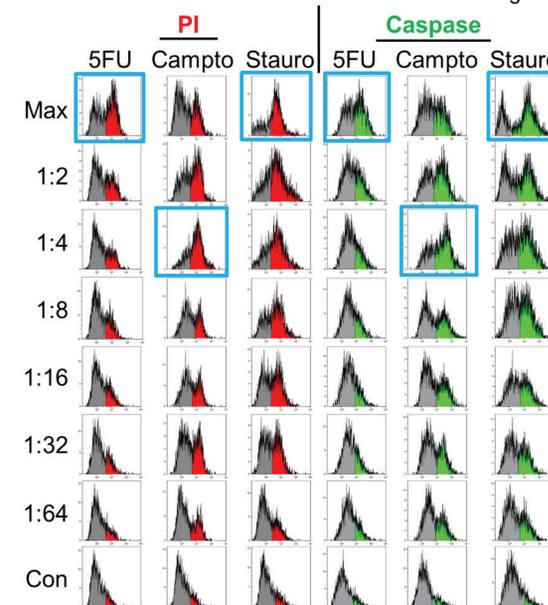


Figure 5. Apoptosis Analysis - Flow Cytometry. Spheroids treated identically as in Figure 4 were dissociated and stained for apoptosis markers for analysis by flow cytometry. Maximal responses (blue boxes) correlate with imaging results for staurosporine and camptothecin but 5-fluorouracil treatment shows significant positive staining (>50% at maximal concentration). This suggests that 5-fluorouracil treated spheroids may be resistant to diffusion of the staining reagents.

Summary

We have automated 3D cell culture in Perfecta3D plates - from plating to spheroid treatment and analysis by imaging and flow cytometry. Automated cell plating resulted in consistent spheroid formation and serial dilutions of compounds gave dose-response information for 3D cultures. By utilizing two different analyses we were able to achieve complementary results. Imaging can provide sphere-level data at multiple time points with minimal sample preparation while flow cytometry can provide cell-level data and reduce the risks of false negatives due to diffusion effects.